In vitro and in vivo models for studying Zika virus biology

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Abstract
The emergence and rapid spread of Zika virus (ZIKV) in the Americas has prompted the development of in vitro and in vivo models to understand several aspects of ZIKV biology and boost the development of vaccines and antivirals. In vitro model studies include reverse genetics systems, two-dimensional (2D) cell models, such as primary cells and cell lines, and ex vivo three-dimensional (3D) models derived from skin, brain and placenta. While these models are cost-effective and allow rigorous control of experimental variables, they do not always recapitulate in vivo scenarios. Thus, a number of in vivo models have been developed, including mosquitoes (Aedes sp. and Culx sp.), embryonated chicken eggs, immunocompetent and immunodeficient mice strains, hamsters, guinea pigs, conventional swine and non-human primates. In this review, we summarize the main research systems that have been developed in recent years and discuss their advantages, limitations and main applications.

INTRODUCTION
Zika virus (ZIKV) is a mosquito-borne arbovirus that has silently circulated in African and Asian countries for many decades and only caused outbreaks of a mild febrile illness. In 2007, however, a large ZIKV outbreak occurred in Yap Island. Later, the virus reemerged in French Polynesia in 2013 and rapidly spread throughout the Pacific [1, 2]. Nonetheless, the dramatic increase of birth defects reported in 2015 in Brazilian newborns changed the world’s perspective on this hitherto overlooked pathogen [3]. Fifty-nine countries and territories have reported ZIKV cases from 2015 onwards. The virus continues to spread geographically to areas where competent vectors are present [4]. In the Americas continentalone, there have been 223 477 confirmed autochthonous cases of Zika disease and 3720 cases of congenital Zika syndrome (CZS) since 2015 [5].

The virus belongs to the genus Flavivirus within the Flaviviridae family and as such it has a single-stranded positive-sense RNA genome that is approximately 11 Kb in length. The genome is translated as a single long open reading frame (ORF) that is flanked by 5’ and 3’ untranslated regions (UTRs) [6]. Translation of the ZIKV ORF generates three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The ZIKV genome produces two types of 3’UTR-derived, non-coding subgenomic flavivirus RNA (sfRNA), which play a role in antagonizing the host innate immune response [7, 8].

ZIKV is primarily transmitted to humans through the bites of infected mosquitoes from certain species. However, the virus can also be transmitted by blood transfusion, transplacentally, perinatally and sexually [9]. In most patients, infection by ZIKV causes a self-limiting exanthematous disease. However, foetuses infected with ZIKV may develop a number of serious teratogenic effects, including microcephaly, cerebral calcifications, ventriculomegaly, cerebellar hypoplasia, arthrogryposis, diaphragm paralysis, and visual and hearing impairments [10]. Moreover, in some patients the virus can cause a broad range of severe neurological manifestations, such as encephalomyelitis, myelitis, ophthalmologic disease, and Guillain–Barré syndrome (GBS) [11–13].

These severe clinical manifestations in humans have prompted the development of several in vitro and in vivo models aimed at uncovering the underlying mechanisms of ZIKV pathogenesis and transmission and boosting the development of countermeasures. In this review, we...
summarize the main research systems that have been developed to study the biology of ZIKV in recent years and discuss their advantages, limitations and applications (Fig. 1).

**IN VITRO MODELS**

**Two-dimensional cell culture models**

Two-dimensional (2D) cell culture systems provide a simple and valuable system for studies in a highly controlled environment. Primary cells are isolated directly from animal or human tissue and usually have a limited lifespan. Conversely, cell lines can be continually passaged over a long period of time, since they have acquired mutations that allow them to proliferate readily. Primary cells better resemble the original tissue from which they were isolated, but they are more difficult to obtain, maintain and propagate compared to cell lines.

**Primary cells**

The infection of primary human epidermal keratinocytes obtained from neonatal foreskins and *in vitro*-generated immature dendritic cells (DCs) resulted in active ZIKV replication and the activation of the innate immune response [14]. Heparinized whole blood and human peripheral blood mononuclear cells (PBMCs) from healthy donors are also susceptible to ZIKV infection. CD14+ blood monocytes are major targets for ZIKV and blood from pregnant women showed an enhanced susceptibility to infection by different ZIKV strains, suggesting differential immunomodulatory responses of blood monocytes during pregnancy [15, 16]. However, these data should be taken with caution, because they are based on studies carried out in countries were dengue is endemic and antibodies against dengue may modulate ZIKV infectivity by an antibody-dependent enhancement (ADE) mechanism [17]. Primary human DCs supported productive ZIKV replication following infection and exhibited donor-dependent variability in viral replication, but not viral binding. Different ZIKV strains antagonized type I interferon (IFN)-mediated phosphorylation of STAT1 and STAT2 [18].

Siemann and coworkers provided insights into the pathogenesis of ZIKV for male hosts by demonstrating that primary human Sertoli cells are susceptible to ZIKV infection.
ZIKV infection of these cells leads to a strong antiviral response which compromises the integrity of the blood-testis barrier [19]. Amniotic epithelial cells (AmEpCs) isolated from human mid- and late-gestation placentas are productively infected by ZIKV. Trophoblast progenitor cells (TBPCs) from chorion, human placental fibroblasts (HPFs) and cytrophoblasts (CTBs) from chorionic villi are also susceptible to ZIKV. Interestingly, cells from mid-gestation produced higher titres than cells from late gestation [20]. Jurado et al. demonstrated that ZIKV productively infects primary human placental macrophages, known as Hofbauer cells, and placental villous fibroblasts [21].

An independent study performed with human placental trophoblasts showed that ZIKV actively replicates in these cells without causing trophoblast dysfunction, senescence or death. This suggests that the placenta may serve as a silent portal for mother-to-fetus transmission [22]. Primary human endometrial stromal cells (HESC) supported the replication of different ZIKV strains. ZIKV replication and expression of the entry co-receptor AXL was enhanced by cyclic AMP and progesterone-induced decidualization of these cells, indicating that they may serve as virus sources for spreading to placental trophoblasts during pregnancy [23]. Primary human trophoblasts (PHTs), which are the barrier cells of the placenta, are refractory to ZIKV infection through the constitutive release of type III IFNα1, which functions in both a paracrine and an autocrine manner to protect trophoblast and non-trophoblast cells from infection [24].

Given the devastating neurological disorders linked to ZIKV affecting the peripheral and central nervous systems (PNS and CNS, respectively), Cumberworth et al. infected primary mouse PNS and CNS myelinating cells derived from wild-type and Ifnar1 knockout mice with ZIKV [25]. Through systematic quantification of ZIKV-infected cells in myelinating cultures, they found that CNS cells are considerably more susceptible to infection than PNS cells, especially CNS axons and myelinating oligodendrocytes. The infection of primary human astrocytes and microglia resulted in high viral replication and the induction of elevated levels of proinflammatory immune cytokines, which may be involved in neuropathogenesis [26, 27]. Primary human neural stem cells (hNSCs) derived from three different donors demonstrated donor-dependent ZIKA-mediated transcriptome alterations and reduction in neuronal differentiation, suggesting that a genetic component is involved in ZIKV neuropathology [28].

**Cell lines**

Viral growth kinetics in cell culture is useful to characterize different viral strains, and also to identify and test drug and vaccine candidates. In general, cell lines are considered to be genetically and phenotypically homogenous, but biological differences among the same cell line from different laboratories might occur and researchers should be aware of this possibility. Like other flaviviruses, ZIKV replicates well in Vero and C6/36 cells, which are widely used for virus isolation from both clinical and mosquito samples [7, 29]. The susceptibility of different human and animal cell lines has been systematically characterized. Here we will summarize a few studies using different cell lines. Human cell lines derived from placenta (JEG-3), neuroines (SF268), muscle (RD), retina (ARPE19), lung (Hep-2 and HFL), colon (Caco-2) and liver (Huh-7) allowed productive ZIKV replication and displayed cytopathic effects (CPE). In contrast, cell lines originating from prostate (LNCaP), testes (833K3), cervix (Hela), endometrium (HOSE6-3) and kidneys (HEK) supported viral replication but did not show CPE. Among animal cell lines, those of non-human primate (Vero and LLC-MK2), swine (PK-15), rabbit (BHK21) and chicken (DF-1) origin permitted productive ZIKV replication [30]. Guinea pig lung fibroblast cells (JH4) are also susceptible to infection [31]. Recently, a human neuroblastoma cell line (SH-SY5Y) has been shown to be very useful for the evaluation of antiviral drugs against ZIKV [32].

ZIKV replicates in human monocytic leukaemia cells (THP-1) at a low rate, but triggers robust antiviral innate cytokine responses. The infection of first trimester human extravillous trophoblast cells (HTR8) demonstrated that ZIKV replicates efficiently in these cells and induces strong inflammatory cytokine and chemokine production [33]. ZIKV can also infect U87-MG (human glioblastoma) cells and produce NLRP3 inflammasome activation and IL-1β release after infection. Increased gene expression for superoxide dismutase 2 (SOD2) and heme oxygenase (HemeOX), two important antioxidant enzymes commonly used to assess oxidative stress, has also been observed. This suggests that ZIKV infection can cause oxidative stress and inflammasome activation, which can lead to cell death via pyroptosis and CNS damage [34].

Mosquito cell lines have been used to elucidate basic biological questions. For instance, Varjak et al. used the *Aedes aegypti*-derived Aag2, AF319 and AF5 cell lines to elucidate ZIKV–mosquito RNAi interactions [35]. C6/36 cells were also employed to investigate the cytoarchitecture of ZIKV during replication, and the authors suggested that these cells are good models for this kind of study [36–38]. To demonstrate ZIKV inhibition by Wolbachia strains in mosquito cell cultures, Schultz et al. used *Ae. albopictus* C710 and C/ wStri cells (derived from C710 cells). In this case, this study confirmed previous data obtained using *in vivo* models [39].

**Three-dimensional (3D) cell culture models**

Although 2D cell culture systems allow better control of experimental variables and are much easier to manipulate than 3D cell cultures, they do not exhibit the natural physiological conditions, cytoarchitecture and cellular complexity present *in vivo*. Therefore, 2D systems sometimes provide misleading or nonpredictive outcomes for *in vivo* settings [40]. Thus, researchers have chosen 3D systems to better characterize infection. Table 1 summarizes the main 3D cell

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**Table 1. Main 3D Cell Culture Models for ZIKV Infection**

<table>
<thead>
<tr>
<th>Model Type</th>
<th>Structure</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organotypic</td>
<td>Organ-like</td>
<td>Better physio-</td>
<td>More complex, difficult to maintain</td>
</tr>
<tr>
<td>spheroids</td>
<td>spheroids</td>
<td>logical conditions</td>
<td></td>
</tr>
<tr>
<td>Monolayer</td>
<td>Single-layer</td>
<td>Easy to manipulate</td>
<td>Limited complexity</td>
</tr>
<tr>
<td>Suspension</td>
<td>Floating cells</td>
<td>Variable morphology</td>
<td></td>
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<tr>
<td>Micromass</td>
<td>Three-dimensional</td>
<td>Enhanced cellular interactions</td>
<td></td>
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**References**


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systems developed to date. Protocols for generating human brain region-specific organoids have been developed [41]. The skin is the site of ZIKV entry following the bite of an infected mosquito. Skin explants obtained from healthy donors following abdominoplasty surgery have proved valuable in understanding ZIKV pathogenesis in this site [14]. Because of their differentiation potential, pluripotent stem cells can generate virtually any cell type. Recently, structures resembling whole organs, termed organoids, have been generated from stem cells through the development of 3D culture systems [42]. Organoid cell culture systems are based on the properties of stem cells to differentiate and self-organize, creating multi-cellular tissues that resemble the structure of and function as an intact organ [43]. Several studies have used this system to study the connection between ZIKV infection and microcephaly [44–49]. Organoids also allow us to understand phenotypic and transcriptomic responses during neural development, for example [45]. ZIKV infects cells at different stages of brain maturation, leading to alterations in the cortical layer organization [47]. Qian et al. [44] engineered a miniaturized spinning bioreactor using 3D design and printing technology and developed protocols to generate forebrain-specific organoids from human iPSCs and for midbrain and hypothalamic organoids. They provided an accessible, cost-effective, simple-to-use and versatile platform for modelling human brain development and disease and for screening antiviral drug candidates. Garcez et al.

### Table 1. Three-dimensional (3D) cell culture models

<table>
<thead>
<tr>
<th>3D model</th>
<th>ZIKV strain</th>
<th>Major findings</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Forebrain-specific neural progenitor cells</td>
<td>MR766 (original ZIKV strain)</td>
<td>ZIKV virus directly infects human cortical neural progenitor cells with high efficiency, resulting in stunted growth of this cell population and transcriptional deregulation</td>
<td>[50]</td>
</tr>
<tr>
<td>Developing human retina progenitors and cerebral organoid model</td>
<td>?</td>
<td>The candidate ZIKV receptor AXL is highly enriched in radial glia, the neural stem cells of the human foetal cerebral cortex, providing a hypothesis for why these cells are particularly vulnerable to ZIKV infection and a candidate mechanism for ZIKV-induced microcephaly</td>
<td>[48]</td>
</tr>
<tr>
<td>Induced pluripotent stem cells (iPSCs) growing as neurospheres and brain organoids</td>
<td>MR766</td>
<td>ZIKV induces cell death in human iPSC-derived neural stem cells (NSCs), disrupts the formation of neurospheres and reduces the growth of organoids</td>
<td>[46]</td>
</tr>
<tr>
<td>Forebrain-specific organoids from human iPSCs</td>
<td>MR766 and FSS13025 (Asian lineage)</td>
<td>Development of a forebrain organoid platform for chemical compound testing and modelling ZIKV infection. ZIKV, upon access to the foetal brain, targets NSCs and causes microcephalic-like deficits in cortical development</td>
<td>[44]</td>
</tr>
<tr>
<td>Human cortical NPCs, neurons, neurospheres and cerebral organoids</td>
<td>ZIKV-BR (patient from Paraiba, 2015) and MR766</td>
<td>ZIKV-BR induces cell death in human NPCs, impairing the growth and morphogenesis of healthy neurospheres. Microcephaly is a distinctive feature of recent ZIKV Asian-lineage virus</td>
<td>[47]</td>
</tr>
<tr>
<td>Explants JEG-3 (cytotrophoblast cell line) and BeWo (cytotrophoblast cell line)</td>
<td>H/PF/2013 (French Polynesia, 2013)</td>
<td>ZIKV infects pregnant dams and placenta, infecting the developing foetus, causing a foetal syndrome that resembles intrauterine growth restriction and spontaneous abortion</td>
<td>[72]</td>
</tr>
<tr>
<td>Chorionic villus explants</td>
<td>MR766, Nica1-16 and Nica 2-16 (isolates from Nicaraguan patients)</td>
<td>ZIKV replicates in primary human placental cells from mid- and late gestation and villus explants from first-trimester human placentas, suggesting placental and paraplacental routes of transmission, and that infection of these cells is inhibited by the cyclic peptide duramycin</td>
<td>[20]</td>
</tr>
<tr>
<td>Primary human Hofbauer cells (HBCs) and cytotrophoblast cells from term placentas</td>
<td>ZIKV-UG/MR766 (Uganda 1947), ZIKV-CAM/FFS13025 (Cambodian/Asian isolate from 2010) and ZIKV-MEX/MEX 2-81 (Americas-derived virus isolated in 2016)</td>
<td>Primary placental-specific fibroblasts and HBCs are permissive for ZIKV replication in isolated cultures in vitro, while HBCs demonstrate susceptibility ex vivo in the context of placental tissues</td>
<td>[21]</td>
</tr>
<tr>
<td>iPSCs and cerebral organoids</td>
<td>ZIKV-UG (VR-1838/Uganda 1947) or ZIKV-PR (VR-1843/Puerto Rico outbreak, 2015)</td>
<td>Loss of AXL gene had no effect on infectivity or virus-mediated cell death in neural progenitors or cerebral organoids</td>
<td>[49]</td>
</tr>
<tr>
<td>First-trimester human maternal decidual tissues grown ex vivo as 3D organ cultures</td>
<td>PRVABC59 (patient from Puerto Rico outbreak, 2015) or MP1751 (mosquitoes from Zika Forest, Uganda, 1962)</td>
<td>Zika virus can replicate in the maternal decidua, identifying the maternal uterine aspect of the human placenta as a likely route of ZIKV transmission to the foetus</td>
<td>[51]</td>
</tr>
</tbody>
</table>
used iPSCs cultured as neural stem cells (NSCs) neurospheres and brain organoids to demonstrate that ZIKV infection induces cell death in human iPSC-derived NSCs, disrupts the formation of neurospheres and reduces the growth of organoids. Tang et al. [50] also used a protocol to differentiate iPSCs into forebrain-specific human neural progenitor cells (hNPCs), which can be further differentiated into cortical neurons, as an in vitro model to investigate whether ZIKV directly infects human neural cells and the nature of its impact. They showed that ZIKV can directly infect hNPCs in vitro with high efficiency and that the infection of these cells led to attenuated population growth through virally induced caspase-3-mediated apoptosis and cell cycle deregulation.

Nowakowski et al. [48] hypothesized that protein expression may be promoting ZIKV entry and infectivity during neurogenesis. They analysed the expression of candidate genes mediating flavivirus entry across single cells from the developing human cerebral cortex and reported the importance of candidate ZIKV receptor AXL in vulnerability to ZIKV infection. In agreement with these findings, Wells et al. [49] studied the effects of AXL deletion and demonstrated that it was not able to protect against ZIKV infection.

ZIKV can replicate in first-trimester human maternal decidual tissues grown ex vivo as 3D organ cultures [51]. In explants of chorionic villi from first-trimester placentas, ZIKV infected proliferating villus cytrophoblasts (CTBs), invasive CTBs and Hofbauer cells in the villus core, and expressed E and NS3 proteins, indicating viral replication [20, 21]. Tabata et al. suggested that ZIKV transmission occurs through placental and paraplacental routes and that the virus spreads from basal and parietal decidua to chorionic villi and amniochorionic membranes [52].

**Reverse genetics systems for ZIKV**

Reverse genetics is a powerful tool that allows important viral properties such as replication, virulence, cell penetration, transmission, host range and the function of coding or non-coding genomic regions to be studied. However, the construction of reverse genetics systems for flaviviruses is often difficult, as it involves multiple cloning of fragments of cDNA. The process is laborious and the difficulties encountered in replicating such clones in bacterial cells can cause viral sequences to be unstable and can even cause toxicity in bacterial hosts [53, 54]. Upon the emergence of ZIKV in the Americas, this technology was employed by several groups using the classical and epidemic strains [55–64]. The main ZIKV reverse genetics systems and their major applications are summarized in Table 2. The first system to be developed used the Cambodian ZIKV FSS13025 strain. Rescued viruses were shown to be highly infectious for Ae. aegypti mosquitoes and virulent to both A129 and AG129 mice, although it was more attenuated than the wild-type virus [55].

The Brazilian ZIKV Paraiba 01/2015 strain and the MR766 prototype of ZIKV have been used recently to generate infectious clones. Their genetic stability was further improved by inserting intron sequences into the NS1 and NS5 genes [56, 65]. ZIKV expressing reporter genes such as luciferase and GFP was proved to be a valuable tool for virus growth and replication analysis, as well as antiviral tests [55, 66]. The introduction of an NS1 K265E mutation significantly increased virus production on Vero cells, which has an impact on vaccine production [63]. Using reverse genetics, it was found that a single serine-to-asparagine substitution (S139N) in the prM protein of ZIKV contributes to foetal microcephaly, demonstrating the power of the system to identify genetic determinants of virulence [67].

A bacteria-free approach that does not require cloning, termed ‘infectious subgenomic amplicons’ (ISA), has been used to recover infectious viruses from PCR products in both mammalian and insect cells [59, 62]. The concept of ISA is based on the production by PCR of three to six overlapping DNA fragments that encompass the entire viral genome. The ZIKV genome is flanked by the CMV promoter and the hepatitis delta (HDV) ribozyme followed by the simian virus 40 (SV40) polyadenylation signal in the 5’ and 3’ ends, respectively. The amplicons are then mixed and transfected directly into susceptible cells to enable virus rescue through as yet unknown in cellulo recombination events. Unlike other bacterium-free approaches, the ISA method does not require any additional steps, such as cloning, propagation of cDNA into bacteria, or even RNA synthesis [59].

Setoh et al. used a modified circular polymerase extension reaction protocol to generate de novo a fully functional ZIKV directly from deep sequencing data. This technique has the advantage of generating infectious virus without the need for prior virus isolation and passaging in cell culture and/or suckling mice, which may result in the accumulation of adaptive mutations that may affect viral phenotypes [64].

More recently, infectious clones have been employed for in vivo and in vitro research on emerging mutations. A reverse genetics system was used to evaluate the effect of the V2634M mutation in NS5, a mutation associated with changes in viral replication efficiency as well as the incidence of microcephaly in Latin America. However, the mutant infectious clone showed no significant change in cell culture replication, and nor did it alter the pathogenesis characteristic and virulence of ZIKV in AG6 mice [68].

In addition to the infectious clones, the use of replicons has also been a useful tool to study viral replication, to investigate the role of specific mutations and to discover novel antiviral drugs. They have the advantage of not being infectious, which makes handling them in the laboratory safer [69, 70]. Taken together, these systems are valuable tools for the discovery of new antiviral compounds and for studying the physiopathology of ZIKV infection.
Table 2. ZIKV reverse genetics systems

<table>
<thead>
<tr>
<th>ZIKV strain (country/year)</th>
<th>Nature of system</th>
<th>Molecular strategy</th>
<th>Major findings</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Paraiba_01/2015 (Brazil/2015)</td>
<td>Full-length ZIKV cDNA inserted into the vector pACNR1811 between the CMV promoter and HDV ribozyme and poly(A) signal/RNA Pol-II terminator</td>
<td>Plasmid toxicity and stability in Escherichia coli (strain MC1061) was improved by inserting an intron sequence after nt position 2711 (NS1 gene) and after nt position 8882 (NS5 gene), respectively. A Vero adaption mutation (C-T) was introduced into nt position 5680 of the ZIKV genome (NS5 gene) to generate ZIKV-NS3m carrying a Ser356Phe substitution in the NS3 protein</td>
<td>The clone replicated efficiently in Vero, neuronal and placental cells. However, virus growth was lower than that for wild-type ZIKV</td>
<td>[56]</td>
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<tr>
<td>MR766 (Uganda, 1947)</td>
<td>Infectious subgenomic amplicon (ISA) fragments cloned into vector pUC57. ZIKV genome was cloned between the CMV promoter and HDV ribozyme and poly(A) signal/RNA Pol-II terminator</td>
<td>Four overlapping fragments covering the full-length viral genomic RNA was used. Two clones were generated and in 1 of them the eGFP gene and the protease 2A were fused in-frame to the first 33 amino acids of the ZIKV C protein</td>
<td>ZIKV expressing eGFP was suitable for viral replication studies in both mosquito and human cells and can be used for screening antiviral molecules and measuring neutralizing antibody titres</td>
<td>[66]</td>
</tr>
<tr>
<td>MR766 (Uganda, 1947)</td>
<td>The pWNII- GFP subgenomic West Nile virus (WNV) replicon plasmid was used as a scaffold. The ZIKV genome was cloned between the CMV promoter and HDV ribozyme and poly(A) signal/RNA Pol-II terminator</td>
<td>The ZIKV construct with the wild-type sequence had large deletions, which we deduced were probably a result of homologous recombination events. This was circumvented by inserting a synthetic intron into NS1, such that the coding sequence would be disrupted in bacteria but splicing in mammalian cells would restore the viral RNA</td>
<td>The infectious clone demonstrated similar growth to the parental virus. The addition of introns to the viral cDNA decreased the toxicity in bacteria, which improves the processes of cloning and rescue of the plasmids</td>
<td>[65]</td>
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<td>FSS13025 (Cambodia/2010)</td>
<td>pCR2.1-TOPO with a T7 promoter and a HDV ribozyme sequence were engineered at the 5’ and 3’ ends of the complete viral cDNA for in vitro transcription and for generation of the authentic 3’ end of the RNA transcript, respectively</td>
<td>Five RT-PCR fragments (A–E) spanning the complete viral genome were individually cloned and assembled into the full-length cDNA of ZIKV. Regions spanning the viral prM-E-NS1 genes were into the low copy-number plasmid pACYC177 to reduce bacterial toxicity. A reporter gene was generated by inserting the fused template capsid luciferase gene and the FMD 2A peptide generated between the C-terminus of the luciferase gene and the ZIKV complete open reading frame</td>
<td>The recombinant virus had reduced replication in cell culture. However, it was virulent in mice and successfully infected Ae. aegypti mosquitoes experimentally. The Luciferase-expressing ZIKV was useful for antiviral screening</td>
<td>[55]</td>
</tr>
<tr>
<td>H/PF/2013 (French Polynesia/2013)</td>
<td>The ISA method was used to recover infectious ZIKV. The viral fragments were flanked at the 5’ and 3’ untranslated regions by the pCMV and the HDR/SV40pA and transfected in three different mammalian cell lines (BHK-21, SW13 and HEK-293 cells)</td>
<td>Three overlapping genome fragments were generated for each virus by PCR. The amplicons were pooled and transfected into permissive cells to generate infectious ZIKV. Exchanging DNA fragments, inter- and intra-lineage chimeric ZIKV were produced. This is a bacteria-free approach, which overcomes the problems with plasmid stability in</td>
<td>Using the ISA method, it was possible to recover recombinant viruses derived from wild-type strains and also intra- and inter-lineage chimeras, and this could be a good tool to study the effect of genetic alterations and specific regions of the ZIKV genome. The replication kinetics of parental and recombinant viruses were essentially similar</td>
<td>[62]</td>
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Table 2. cont.

<table>
<thead>
<tr>
<th>ZIKV strain (country/year)</th>
<th>Nature of system</th>
<th>Molecular strategy</th>
<th>Major findings</th>
<th>Reference</th>
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<tbody>
<tr>
<td>H/PF/2013 (French Polynesia/2013) taylori-tc/SEN/1984/41662-DAK (Senegal/1984)</td>
<td>The ISA method was used to recover infectious ZIKV. Virus was rescued in both mosquitoes (C6/36) and mammalian (BHK-21) cells. The ZIKV genome is flanked in 5’ and 3’ by the CMV promoter and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal, respectively.</td>
<td>Three fragments comprising the whole genome were transfected to generate an infectious clone. Three overlapping linear non-infectious subgenomic DNA fragments that encompass the entire viral genome were mixed and transfected directly into susceptible cells to enable the recovery of infectious viruses. This is a bacteria-free approach, which overcomes problems with plasmid stability in bacteria.</td>
<td>The ISA method was used to produce recombinant viruses in mammalian cells and mosquitoes efficiently; in addition to ZIKV, other arboviruses (YFV, JEV, CHIKV and WNV) were also efficiently recovered by this methodology.</td>
<td>[59]</td>
</tr>
<tr>
<td>SPH2015 (Brazil/2015) and SZ-WIV0 (China/2016)</td>
<td>pFK-Jc1E2Flag vector. Artificial synthetic DNA sequences that contain ZIKV cDNA flanked by T7 promoter and HDV ribozyme were cloned into pFK plasmid, which is a low-copy vector</td>
<td>Several silent mutations were inserted into the viral genome sequence in order to reduce the activity of cryptic bacterial promoters from the infectious clone and thus facilitate rescue of the plasmid.</td>
<td>The plasmid was stable in bacteria, and the recombinant ZIKV virus was able to infect a panel of cell lines, including Vero E6, C6/36, U-251MG, and cause lethal infection in AG6 mice. The V2634M substitution in NS5 caused negligible effects for the ZIKV life cycle in cell culture and pathogenesis in AG6 mice.</td>
<td>[68]</td>
</tr>
<tr>
<td>GZ01 (China/2016)</td>
<td>Four ZIKV fragments were cloned into plasmids vectors. The SP6 promoter was placed upstream of the 5’ end of the ZIKV genome</td>
<td>The construction was engineered to contain a modified version of the group II self-splicing intron P.ii.PSU12 near the junction between the E and NS1 genes, which were removed from the RNA transcripts by an in vitro splicing reaction. Spliced viral RNA transcripts were used to generate infectious virus upon transfection of susceptible cells.</td>
<td>Self-splicing ribozyme-based construction abolished the potential toxicity of ZIKV cDNA clones to the E. coli host. Two crucial cis-acting replication elements (5’-SLA and 5’-CS) of ZIKV were identified [60] and a S139N mutation in the pM protein of ZIKV was found to contribute to foetal microcephaly [67].</td>
<td>[64]</td>
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<tr>
<td>Natal RGN (Brazil/2015)</td>
<td>Seven DNA fragments covering the entire genome were generated by circular polymerase extension reaction (CPER) and cloned into the pUC19 vector. An eighth pUC19 plasmid contained elements required for transcription (CMV, HDV ribozyme and a polyadenylation (pA) signal)</td>
<td>These plasmids were then used to generate cDNA fragments by PCR, with the resulting eight dsDNA fragments being mixed in equimolar amounts and subjected to cycles of CPER with a high-fidelity polymerase. The CPER products were then transfected into Vero cells for viral rescue.</td>
<td>Fully functional Zika virus isolates were obtained directly from deep sequencing data from virus-infected tissues without the need for prior virus passaging and for the generation and propagation of full-length cDNA clones. The recombinant ZIKV was virulent for mice foetuses and transmitted efficiently in Ae. aegypti mosquitoes.</td>
<td>[64]</td>
</tr>
<tr>
<td>PRVABC59 (Puerto Rico/2015)</td>
<td>The ZIKV genome was cloned in two separate pieces into the pACYC177 vector using Gibson assembly. The ZIKV genome was flanked by a T7 promoter and an HDV ribozyme sequence.</td>
<td>Plasmids were amplified, digested and ligated using Gibson assembly to create a plasmid containing the full-length virus sequence with a T7 promoter and hepatitis D virus ribozyme sequence, which was then transcribed into RNA using T7 polymerase. The resulting RNA was then electroporated into Vero cells to generate infectious virus. A single synonymous mutation was identified at nucleotide position C8489T,</td>
<td>Rescued ZIKV replicated similarly to wild-type virus in both human and mosquito cells. Infectious clone-derived virus initiated displayed similar rates of transmission in Ae. aegypti mosquitoes and similar pathogenesis in AG129 mice as compared to the wild-type virus.</td>
<td>[57]</td>
</tr>
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### Table 2. cont.

<table>
<thead>
<tr>
<th>ZIKV strain (country/year)</th>
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<th>Molecular strategy</th>
<th>Major findings</th>
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<tr>
<td>MR766 (Uganda, 1947) H/PF/2013 (French Polynesia/2013) PRVABC59 (Puerto Rico/2015) BeH819015 (Brazil/2015) SPH2015 (Brazil/2015)</td>
<td>The genome of the virus is divided into four fragments using the diagrammed restriction endonucleases and cloned into pUC37 vector. ZIKV genome was flanked by a T7 promoter and the HDV ribozyme. Plasmids were amplified in E. coli strain MC1061</td>
<td>which allowed differentiation from wild-type virus The four plasmids were digested, ligated, in vitro-transcribed and electroporated into C6/36 cells to rescue virus</td>
<td>A panel of six infectious clones was generated. This system allowed the identification of lethal errors in the published sequence of the SPH2015 virus. Recombinant viruses had satisfactory replication in cell culture, immunogenicity and virulence in mice</td>
<td>[58]</td>
</tr>
<tr>
<td>FSS13025 (Cambodia/2010)</td>
<td>The ZIKV replicon was constructed by replacing the viral structural genes with Rluc using a full-length cDNA infectious clone (pFLZIKV) obtained from a previous study [55]. A T7 promoter and an HDV ribozyme sequence were engineered at the 5' and 3' ends of the replicon cDNA for in vitro transcription and for generation of the authentic 3' end of the RNA transcript, respectively</td>
<td>The C38-Rluc2A-E30 cassette encodes the N-terminal 38 amino acids of protein C, Rluc reporter, FMDV 2A protease and the 30 C-terminal amino acids of the E1 protein (E30) of ZIKV. The cassette was cloned into pFLZIKV at the NotI and SphI sites, replacing the structural genes. A second ZIKV replicon containing Rluc and Neo genes (Rep-Neo) was constructed by inserting an EMCV IRES into the 3' UTR of the above Rluc replicon to generate a stable cell line expressing ZIKV proteins</td>
<td>The replicons were used to evaluate the effect of the NS5 mutation on viral RNA synthesis and the analysis of a known inhibitor of ZIKV. The replicon was useful for antiviral screening without the use of infectious virus, since the clones do not generate infectious particles, eliminating the risk of viral infection acquired in the laboratory</td>
<td>[70]</td>
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### IN VIVO MODELS

#### Murine models

Murine models have contributed significantly to the acquisition of new insights into the biology of ZIKV infection [47, 71–73]. Mice have contributed to elucidate several aspects of ZIKV pathogenesis, including the link between ZIKV infection in pregnant women and congenital defects [46, 50, 74]. Mice have also been a valuable model for the evaluation of vaccine and antiviral candidates. Their small size, low cost and fast reproductive rate make these animals attractive models for ZIKV studies.

#### Immunocompetent mice strains

Immunocompetent adult mice show little susceptibility to ZIKV infection and do not emulate the spectrum of ZIKV clinical manifestations seen in humans (Table 3). One of the reasons for this phenotype is the ability of ZIKV to degrade STAT2, an IFN-regulated transcriptional activator in humans but not mice [75]. Since ZIKV is sensitive to the action of IFN types I, II and III, blockade of IFN receptors has been used to develop susceptible mouse models [14, 24, 76]. Lazear *et al.* used an IFNAR1-blocking monoclonal antibody (MAR1-5A3) in C57BL/6 mice to make them susceptible to ZIKV infection. These animals did not develop neurological manifestations and the disease was not as severe as that observed in Ifnar1<sup>−/−</sup> mice [76]. A similar approach was applied to develop a lethal C57BL/6 mice model. In this study, animals inoculated with the DAKAR D41525 strain via either the subcutaneous or the intraperitoneal route had 40 and 100 % mortality rates, respectively. ZIKV administration through either route caused viraemia, motor impairment and weight loss [77]. Immunocompetent mice treated with anti-IFNar1 antibodies have also been used for the discovery of antiviral drugs acting against ZIKV. Five-week-old CB57/6 mice previously treated with anti-ifnar1 antibodies and later infected with the mouse-adapted DAKAR 41519 strain showed a survival rate of almost 20 %, even when not treated with sofosbuvir, an inhibitor of the ZIKV NS5 RNA-dependent RNA polymerase (RdRp) [78]. The DAKAR D41519 strain is a lethal mouse-adapted ZIKV strain obtained following brain homogenate passage in Rag1<sup>−/−</sup> mice [79]. The antiviral action of sofosbuvir was also demonstrated in 3-day-old Swiss mice, without the need for IFN blockage [80].

Given that mouse models based on antibody blockade of IFN receptors are expensive for routine use and may not be able to completely deplete the IFN response [78], alternative ways to develop immunocompromised models must be explored. One strategy is to administer immunosuppressive drugs. After ZIKV infection, dexamethasone-immunocompromised BALB/c mice sustained high viral replication in multiple organs, inflammation and mild weight loss after inoculation via the peritoneal route [81]. Interestingly, immunohistochemical analyses conducted in the tissues of...
lenged intracranially with the MR766 ZIKV strain, they also to the viral strain used. When CD1 mice were chal-
lenge were partially due to the host immune response to
the placenta and foetal brain [90].

Previous studies have indicated the possible protective role of T-cell responses during ZIKV infection [82]. SJL mice are immunocompetent, but have elevated levels of circulating T-cells [83, 84]. Interestingly, pregnant SJL mice, when inoculated intravenously with a high dose of 10^{10}–10^{12} plaque-forming units (p.f.u.) of ZIKV, generated pups that presented neurological and ophthalmological malformations similar to those observed in humans, in addition to intrauterine growth retardation [47]. Using C57BL/6 mice, the authors found that ZIKV was not able to cross the placental barrier in C57BL/6 females infected during gestation, as they had offspring that were free of infection [47]. In contrast, foetuses of C57BL/6 female mice that were inoculated intraperitoneally with an Asian lineage strain during gestation displayed signs of ZIKV infection in their brains and viral RNA was detected in mouse placetas at day 3 post-inoculation [85].

Similar to C57BL/6 mice, the outbred CD-1 mice have been demonstrated to be resistant to ZIKV infection. Infection of these animals at 3 weeks with the FSS13025 ZIKV Asian strain resulted in no clinical signs of infection and no detectable viraemia [73]. However, this apparent resistance might not be solely due to the route of infection chosen, but also to the viral strain used. When CD1 mice were challenged intracranially with the MR766 ZIKV strain, they exhibited an 80–100 % mortality rate that was age-independent. Intraperitoneal inoculation with the same strain generated morbidity signs, but no mortality in a dose-dependent manner [86]. Remarkably, 5–6-week-old female 129 Sv/Ev mice inoculated through the subcutaneous route with 10^{6} pfu of ZIKV showed no clinical and histopathological signs of infection. Viraemia and low viral RNA levels were found in the brain, ovaries, liver and spleen after infection [87]. Remarkably, intravenous ZIKV infection of pregnant immunocompetent C57BL/6/ pregnant mice led to profound placental pathology and a high frequency of foetal demise, in the absence of foetal infection [88].

Another immunocompetent mouse used in ZIKV research is the ICR strain, a highly prolific outbred mouse strain derived from Swiss mice. Direct intracerebral inoculation of mouse foetuses resulted in high viral replication, cell cycle arrest, apoptosis and inhibition of neural precursor cell differentiation, leading to microcephaly [89].

A fully immunocompetent humanized mouse model has been recently developed by knocking in (KI) the human STAT2 into the mouse Stat2 locus (hSTAT2 KI). Infection of pregnant hSTAT2 KI mice was carried with a highly virulent mouse-adapted ZIKV strain derived from the ZIKV-Dak-41525 strain. The infection resulted in viral spread to the placenta and foetal brain [90].

In addition to mouse age and lineage, the route of infection can directly affect the outcome of ZIKV infection. Signifi-
cant lesions and cell death in the CNS were observed in newborn Swiss mice inoculated with ZIKV through either

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Age</th>
<th>ZIKV strain (country/ year)</th>
<th>Route of infection/dose</th>
<th>Major findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6f</td>
<td>5 weeks old</td>
<td>Dakar 41519 (Senegal/1984)</td>
<td>Subcutaneous/10^{6} p.f.u.</td>
<td>Mice infected after treatment with antibody had a better rate of survival after the FDA-approved drug sofosbuvir was administered. However, almost 20 % of the untreated mice survived ZIKV infection</td>
<td>[78]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>5–8 weeks old</td>
<td>PRVABC59 (Puerto Rico/2015)</td>
<td>Intraperitoneal/3.24×10^{6} p.f.u.</td>
<td>High viral load was observed in dexamethasone immunosuppressed mice, as well as the development of several inflammations in organ tissues. Treatment with recombinant type I interferons was able to mitigate disease effects</td>
<td>[81]</td>
</tr>
<tr>
<td>129 Sv/Ev</td>
<td>5–6 weeks old</td>
<td>MP1751 (Uganda/1962)</td>
<td>Subcutaneous/10^{6} p.f.u.</td>
<td>Low viraemia; no weight loss; no clinical disease; viral detection only in the brain, ovaries, spleen and liver</td>
<td>[87]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5 weeks old</td>
<td>DAK AR D 41525 (Senegal/1984)</td>
<td>Subcutaneous or intraperitoneal/10^{6} p.f.u.</td>
<td>Anti-Ifnar1-treated mice suffered severe lesions to CNS; high viraemia; weight loss; intraperitoneal injection led to a 100 % mortality rate</td>
<td>[77]</td>
</tr>
<tr>
<td>Swiss</td>
<td>1 day old</td>
<td>SPH 2015 (Brazil/2015)</td>
<td>Subcutaneous/10^{6} p.f.u.</td>
<td>Mild to severe CNS lesions observed; ataxia; paralysis; lethargy; subcutaneous infection caused spinal cord damage and myelopathy</td>
<td>[91]</td>
</tr>
<tr>
<td>FBV/NJ and C57BL/6f</td>
<td>5.5, 7.5,9.5 or 12.5 days post-coitum</td>
<td>HS-2015-BA-01 (Brazil/2015)</td>
<td>Intravenous (jugular vein)/10^{6} p.f.u.</td>
<td>Earlier infections lead to severe malformations and intrauterine growth restriction; 10.5 days post-challenge (p.c.) the exposed mice developed both dysraphia and hydrocephalus; no significant effects noticed after 12.5 days p.c.</td>
<td>[92]</td>
</tr>
<tr>
<td>SJL</td>
<td>10–13 embryonic days</td>
<td>ZIKVBR (Brazil/2015)</td>
<td>Intravenous/10^{5}, 4×10^{5}, 10^{5} p.f.u.</td>
<td>Neurological and optical malformations; intrauterine growth restriction; upregulation of apoptotic related genes</td>
<td>[47]</td>
</tr>
</tbody>
</table>

Table 3. Immunocompetent mice models for studying ZIKV biology
the intracerebral or the subcutaneous route, which led to the development of myelopathy and encephalopathy, respectively. Further, animals inoculated by the subcutaneous route presented spinal cord injury [91]. A haematogentic infection model was conceived to evaluate the effects of ZIKV infection on embryonic and foetal development, using FBV/NJ and C57BL/6 mice. Early infections caused growth restriction and/or severe malformations in infected embryos, including hydrocephalus and dysraphia. Later exposure to ZIKV did not generate significant effects as foetal development progressed [92]. Remarkably, intravenous ZIKV infection of pregnant immunocompetent C57BL/6J pregnant mice led to profound placental pathology and a high frequency of foetal demise, in the absence of foetal infection. In this study, placental pathology rather than embryonic/foetal viral infection seemed to be a stronger contributor to adverse pregnancy outcomes in mice and direct viral infection of the embryo was not essential for foetal demise. [88]. Yockey et al. observed long-lasting infections and high rates of viral replication in C57BL/6 females infected intravaginally. In addition, foetuses of females inoculated by this route during pregnancy developed cerebral infection and intruterine growth retardation [93].

Thus, although immunocompetent mice models present limitations regarding clinical manifestation of the disease, they are valuable to obtain evidences about viral pathogenesis under the full action of the innate and adaptive immune responses of the host to ZIKV.

**Immune-deficient strains**

In contrast to immunocompetent mice, immunocompromised mice display signs of disease and high levels of viraemia, and can be infected even with low-passage viral strains (Table 4). The major disadvantage of immunocompromised mice is the lack of essential components of the immune response, which may underestimate the efficacy of some vaccine candidates and not model disease pathogenesis accurately in immunocompetent hosts. Nevertheless, these models have been successfully used for preclinical evaluation of vaccines and antivirals against ZIKV [94–97]. A129 mice lack the receptor for IFNα/β, making them unresponsive to type I IFNs. It was one of the first models used to characterize ZIKV infection, although it had already been used in studies about other viruses, such as chikungunya virus (CHIKV) and yellow fever virus (YFV) [87, 98]. On the other hand, the AG129 mouse strain is a double knockout for the IFNα/β and γ receptors and is more susceptible to ZIKV-induced disease than A129 mice, highlighting the role of IFN-γ in the outcome of the disease. Although the ZIKV infection kinetics in AG129 mice is similar to that in A129, the disease signs are more severe in AG129, probably because of the protective role of IFNγ [57, 71–73].

The A129 mice may also be useful in vaccine challenge studies since they remain susceptible to the induction of morbidity and mortality caused by ZIKV even when infected at 6 months of age. The detection of persistent infection foci in organs such as the brain, spinal cord, testes and ovaries even after the resolution of disease symptoms is an important finding in the study of the development of GBS and congenital infections (associated or not with microcephaly) and the occurrence of sexual transmission [73, 76, 87, 99]. Furthermore, Rossi and colleagues [73] characterized ZIKV infection in both A129 and AG129 mice. Intraperitoneal ZIKV infection of A129 mice resulted in clinical disease (tremors, lethargy and anorexia) and mortality in an age-dependent manner. The virus replicated in several organs, but the highest titres were found in the spleen, testes and brain. Overall, little difference was seen between the disease and virulence of ZIKV in A129 and AG129 mice, except for the severity of the neurological manifestations, which was more pronounced in the latter strain [73]. The neuroviremulence of ZIKV in AG129 mice was endorsed by Aliota and coworkers, who reported significant histopathological lesions in the brain upon infection [71]. These observations suggest the relevance of these mice strains for studying disease pathogenesis in humans, including the Guillain–Barré Syndrome and microcephaly.

Another relevant model is characterized by Irf3−/− Irf5−/− Irf7−/− mice that are C57BL/6 triple knockout (TKO) for

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<tr>
<td>A129</td>
<td>129 Sv/Ev</td>
<td>IFNs and β receptors</td>
<td>Study of the spread of the virus in different organs. Evaluation of the immune response induced by vaccines</td>
<td>[118].</td>
</tr>
<tr>
<td>AG129</td>
<td>129/Sv</td>
<td>IFNα/β and γ receptors</td>
<td>Viral dissemination profile, lethality, mechanisms of neuropathogenesis and attenuation/virulence of viral strains using various inoculation routes</td>
<td>[57, 71–73].</td>
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<tr>
<td>Irf3−/Irf5−/Irf7−/Ift1−/Ift8−/Ift10−/Ift20−/Ift22−</td>
<td>C57BL/6</td>
<td>Transcription factors 3, 5 and 7 (interferon pathway)</td>
<td>Analysis of viraemia and lethality conferred by ZIKV. Important for findings related to infection of neural stem cells</td>
<td>[76, 100].</td>
</tr>
<tr>
<td>LysMCremIrf5−/Ift20−/Ift22−</td>
<td>C57BL/6</td>
<td>IFNα and IFNβ receptors</td>
<td>Characterization of infection. Mimics immunosuppressed individuals, neonates and elderly. Studies on placental infection, trans-placental transmission, neuroinvasion and consequences of neurological disease</td>
<td>[104].</td>
</tr>
<tr>
<td>Rag1−/−</td>
<td>C57BL/6</td>
<td>Type I IFN in myeloid lineage cells</td>
<td>Demonstration of protection mediated by CD8+ T cells in the control of ZIKV infection. Promising for more complete studies on the cellular immune response and for the evaluation of control measures such as vaccines and antivirals</td>
<td>[108].</td>
</tr>
<tr>
<td>LysMCremIrf5−/</td>
<td>C57BL/6</td>
<td>T-cell and B-cell responses</td>
<td>Role of the adaptive cellular response in the control of ZIKV infection</td>
<td>[169].</td>
</tr>
</tbody>
</table>

Table 4. Major immunocompromised mouse strains used in the study of ZIKV infection
the transcription factors interferon-regulatory factors 3, 5 and 7, respectively [76, 100]. This lineage has been used to study the impact of ZIKV in the CNS, where high levels of viral RNA were detected in tissues after infection and severe signs of neurological disease, such as hindlimb weakness and paralysis, were observed [76]. Compared to Ifnar1−/−, which is knockout for IFNα/β receptors, the TKO model was more susceptible to ZIKV infection and this difference points to a role for an IRF-3 dependent, IFN-α/β-independent mechanism. In fact, ZIKV infection blocks the induction of type I IFN by downregulating IRF3 and antiviral NF-κB-mediated signalling and targets STAT2 for proteasomal degradation [101].

Following the discovery that ZIKV evades the innate immune system by targeting STAT2 for degradation, Tripathi et al. infected Stat2−/− mice subcutaneously and showed that this strain is highly susceptible to ZIKV infection. They demonstrated that ZIKV spread systemically in this knockout mice strain and caused neurological disease [102]. Interestingly, it has been observed that SCID mice (deficient in T and B lymphocytes) develop more severe disease and are more resistant to infection than the AG129 model, suggesting that ZIKV infection in mice is mainly controlled by the innate immune response mediated by IFNs rather than the adaptive response mediated by T-cells. A systemic inflammatory response mediated by proinflammatory cytokines was also detected in the sera of AG129 mice [99, 103]

Different immunocompromised mice have also been applied to identify which cells of the nervous system are permissive to replication. Brain analysis of Ifnar1−/− mice revealed that astrocytes are one of the most susceptible types, while cerebral cortex neurons are the least permissive, being observed not only in the brain but also in other regions of the CNS, such as the cerebellum and spinal cord [104]. While in the Ifnar1−/− model the most permissive infection sites were the astrocytes, in Rag1−/− mice treated with anti-IFNAR, the most evident focal areas were neurons in the cerebral cortex and hippocampus regions [105]. The disagreement of these results reinforces the need to explore different models in order to characterize a strain that can mimic the infection in the CNS with more fidelity.

Case reports of ZIKV sexual transmission and viral persistence in the human genital tract are mounting. Murine models recapitulate these phenotypes and may provide bases for understanding the sexual transmission of ZIKV in humans. In fact, recurrent viral detection in the mouse testis has been achieved in experimental studies [73, 76, 106, 107]. In this sense, studies using females may also be informative for research about this route. Vaginal exposure of pregnant mice during early pregnancy resulted in foetal infection and intrauterine growth restriction [93]. The evaluation of different mice strains with attenuation of the innate immune response mediated by IFNs shows that the transcription factors IRF3 and IRF7 are required to block viral replication locally. In contrast, the adaptive immune response does not seem to play a critical role in the control of vaginal infection [93]. Tang and colleagues inoculated ZIKV into the vagina of both AG129 mice and LysMCre+IFNAR−/−C57BL/6 mice, which lack IFNAR in myeloid cells, in diestrus-like and estrus-like phases after respective hormonal treatments [progesterone and pregnant mare serum gonadotropin (PMSG), respectively]. Whereas the mice infected during the estrus-like phase were resistant to vaginal infection, those infected during the diestrus-like phase developed disease following atrumatic intravaginal ZIKV administration. There was a strain-dependent susceptibility, in which LysMCre+IFNAR−/−C57BL/6 mice experienced transient illness and AG129 mice succumbed to infection [108]. These models of venereal transmission will be useful to understand the pathogenesis of ZIKV through this route and will be a suitable challenge system for evaluating the protective efficacy of vaccine and antiviral candidates.

**Chicken embryos**

The chicken embryo is a well-established model in developmental biology and has advantageous features, including size, low cost, easy manipulation and the fact that it allows high-throughput in vivo screening of drugs. The model closely mirrors human foetal neural development and the sequencing of the chicken genome has opened up possibilities for uncovering the molecular basis of development and changes associated with viral infections [109, 110].

An early study using the MR766 ZIKV strain showed that cultured primary embryonic chicken cells were not susceptible to infection [111]. However, recent studies have demonstrated that the DF-1 chicken fibroblast cell line [30] and chicken embryos are susceptible to infection by contemporary ZIKV strains [110]. The infection of chicken embryos at embryonic days 2.5 and 5 resulted in dose-dependent mortality, virus replication in various organs, stunted brain growth and other malformations, such as enlarged ventricles, but not calcifications (Table 5). Thus, the chicken embryo proved to be a well-characterized, non-immunocompromised in vivo animal model capable of recapitulating some of the teratogenic manifestations of ZIKV in human foetuses [110]. The postnatal effects of ZIKV infection of chicken embryos warrant further investigation.

**Guinea pigs**

Guinea pigs have been used as a model for infectious disease since the nineteenth century, including tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) [112–114]. Their small size and docility and the low cost of acquiring and maintaining them make them an attractive laboratory animal model, although the paucity of available immunological reagents is a limitation [115]. In contrast to mice and rats, they have a haemochorial placenta, through which maternal and foetal circulation is separated by a single layer of trophoblasts, making them useful for studying congenital infections [31]. Initial studies characterizing the pathogenicity of the MR766 strain shortly after the discovery of ZIKV by the
virologist George Dick found that guinea pigs \( n=2 \) that had been inoculated intracerebrally had no signs of infection but died on the sixth day after inoculation with a low-passage mouse-adapted virus. Infection with highly mouse-adapted viruses did not result in lethal infection, suggesting that adaptation to mice reduces the virulence to guinea pigs (and also monkeys) [116].

The guinea pig model has been recently revisited using contemporary ZIKV strains (Table 5) [31, 117, 118]. The infection of juvenile animals (200–250 g) with ZIKV PRVABC59 strain (Puerto Rico/2015) via the subcutaneous route resulted in fever, lethargy, hunched back, ruffled fur and decreased mobility. Viraemia was detected at 2 and 3 days post-infection (p.i.), but not at 5 days p.i. ZIKV RNA load was detected in the spleen (only at 2 days p.i.) and brain (at 2, 3 and 5 days p.i.) of these animals. Analysis of cytokines, chemokines and growth factors in the serum using multiplex immunoassay showed a marked increase in the levels of IL-2, IL-5, IL-18, IL-12 (p70), TNF-α, G-CSF, MCP-1, MIP-1α, LIX, fractalkine and VEGF, whose roles in ZIKV pathogenesis need to be further investigated [117]. Deng and coworkers [118] demonstrated that guinea pigs are susceptible to infection through the intranasal route and that the virus can be detected in the sera, saliva, tears, brain and parotid glands. Interestingly, the 2010 Cambodian ZIKV strain FSS13025 was less virulent than the 2016 Chinese ZIKV strain GZ01 FSS13025. Remarkably, close-contact transmission experiments showed that ZIKV is highly transmissible to co-caged animals. No overt clinical signs were

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Age</th>
<th>ZIKV strain (country/year)</th>
<th>Route of infection/dose</th>
<th>Major findings</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Guinea pigs</td>
<td>200–250 g*</td>
<td>PRVABC59 (Puerto Rico/2015)</td>
<td>Subcutaneous/−10⁶ p.f.u.</td>
<td>Fever; lethargy; hunched back; ruffled fur; decreased mobility; seroconversion; viraemia; increase in the levels of multiple cytokines; chemokines and growth factors in the serum Infectious dose 50 % (ID₅₀) of ZIKV was calculated to 10⁻³⁵ p.f.u. by the subcutaneous route; no overt clinical signs; sustained viraemia; viral shedding in both saliva and tears; seroconversion; replication and pathology in multiple organs; 100 % contact transmission</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>5 weeks old</td>
<td>GZ01 (China/2016) or FSS13025 (Cambodia/2010)</td>
<td>Subcutaneous, intranasal, or contact /−10⁶ p.f.u.</td>
<td>Non-pregnant animals had low-level viraemia and no clinical disease; pregnant animals had no overt clinical signs; no interference with normal pup development; no detectable viraemia and antigenemia in pregnant animals; seroconversion in pup and dams</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>6 months old , at 18 to 21 days gestational age</td>
<td>H/PF/2013 (French Polynesia/2013)</td>
<td>Subcutaneous/10⁷ p.f.u.</td>
<td>Viraemia was detected at 2 and 3 days post-infection (p.i.), but not at 5 days p.i. ZIKV RNA load was detected in the spleen (only at 2 days p.i.) and brain (at 2, 3 and 5 days p.i.) of these animals. Analysis of cytokines, chemokines and growth factors in the serum using multiplex immunoassay showed a marked increase in the levels of IL-2, IL-5, IL-18, IL-12 (p70), TNF-α, G-CSF, MCP-1, MIP-1α, LIX, fractalkine and VEGF, whose roles in ZIKV pathogenesis need to be further investigated [117]. Deng and coworkers [118] demonstrated that guinea pigs are susceptible to infection through the intranasal route and that the virus can be detected in the sera, saliva, tears, brain and parotid glands. Interestingly, the 2010 Cambodian ZIKV strain FSS13025 was less virulent than the 2016 Chinese ZIKV strain GZ01 FSS13025. Remarkably, close-contact transmission experiments showed that ZIKV is highly transmissible to co-caged animals. No overt clinical signs were</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>8 weeks old</td>
<td>FSS13025 (Cambodia/2010)</td>
<td>Subcutaneous or intradermal/−10⁶ p.f.u.</td>
<td>No clinical disease; no viraemia; no seroconversion</td>
<td>[121]</td>
</tr>
<tr>
<td></td>
<td>6–8 weeks old</td>
<td>P6-740 (Malaysia/1966)</td>
<td>Subcutaneous/5000, 500, and 50 TCID₅₀</td>
<td>Used STAT2 knock-out hamsters; death; weight loss; viral load in the brain, tests, spinal cords, kidney, spleen; no histopathological lesions; infection of pregnant STAT2 knock-out hamsters led to ZIKV infection in placental and foetal brains, but no detectable phenotype in pups; infection of wild-type hamsters did not result in placental or foetal brain infection</td>
<td>[122]</td>
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<tr>
<td>Hamsters</td>
<td>8 weeks old</td>
<td>MEX 1–44 (Mexico/2015)</td>
<td>In ovo/0.2 to 10⁶ p.f.u.</td>
<td>Embryo mortality; virus replication in various organs; microcephaly-like phenotype; ventriculomegaly</td>
<td>[110]</td>
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<tr>
<td></td>
<td>3 months old</td>
<td>PRVABC59 (Puerto Rico/2015)</td>
<td>Intracerebral/−10⁶ TCID₅₀</td>
<td>Seroconversion and low-titre viraemia, viruria and virus replication in internal organs. Two out of eleven pigs exhibited leg weakness, ataxia and tremor</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td>18 to 21 days gestational age</td>
<td>MEX 1–44 (Mexico/2015)</td>
<td>Subcutaneous or intradermal/−10⁶ p.f.u.</td>
<td>Subclinical infection without viraemia but with seroconversion</td>
<td>[121]</td>
</tr>
<tr>
<td>Chicken embryo</td>
<td>Embryonic days 2.5 and 5</td>
<td>PRVABC59 (Puerto Rico/2015)</td>
<td>In utero intraperitoneally + intra amniotic or intracerebral /−10⁶ TCID₅₀</td>
<td>Detection of ZIKV RNA in foetal membranes; persistent infection in porcine conceptuses; no brain lesions or histopathology in offspring; impaired health in offspring; weak piglets, delayed feeding, neonatal body length and weight lower than controls, seizures, splayed back leg, reduced growth rate, aggressive behavior</td>
<td>[125]</td>
</tr>
<tr>
<td>Swine</td>
<td>1 day old</td>
<td>FSS13025 (Cambodia/2010); PRVABC59 (Puerto Rico/2015)</td>
<td>Subcutaneous or intradermal/−10⁶ p.f.u.</td>
<td>Microcephaly in 2 out of 15 pigs inoculated in utero and mild to severe neuropathology, characterized by neuronal depletion in the cerebral cortices of various lobes, in all foetuses</td>
<td>[126]</td>
</tr>
</tbody>
</table>

*Corresponds to age between 11–13 days.

Table 5. Non-murine models for studying ZIKV biology

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Pena et al., Journal of General Virology 2018;99:1529–1550

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seen for any of the routes of infection used (intranasal, subcutaneous or direct contact). A third study evaluated the effect of ZIKV infection in non-pregnant and pregnant adult guinea pigs infected at mid-gestation (at 18 to 21 days gestational age) [31]. Clinical signs of disease were not observed in either non-pregnant or pregnant animals infected with the 2013 French Polynesian ZIKV strain. Naturally circulating ZIKV strains are not pathogenic to immunocompetent guinea pigs and do not interfere with normal pup development. Although pregnant guinea pigs and their pups seroconverted upon infection, no detectable viral levels were seen in maternal or pup blood, plasma or tissues. Of special interest, no significant adverse outcome was detected in the guinea pigs’ offspring following challenge, suggesting that ZIKV is not pathogenic during the pregnancy of guinea pigs and does not interfere with normal foetal development in this species. The reason for the difference in overt clinical disease among these studies is not clear but might be related to the age of animals used and strain differences.

Hamsters
Syrian golden hamsters (Mesocricetus auratus) have been established as a model for some neurotropic flaviviruses, including WNV [119] and JEV [120]. Recently, the model has been evaluated for ZIKV (Table 5). Like guinea pigs, hamsters are small and relatively inexpensive to maintain, but there are few immunological reagents for this species. The infection of hamsters with a 2010 Cambodian ZIKV strain did not result in clinical disease, viraemia or seroconversion [121]. However, genetic ablation of the STAT2 gene, a key mediator of type I and type III IFN signal transduction pathway signalling, renders these animals susceptible to ZIKV infection [122]. Subcutaneous infection with 500 or 50 TCID50 of ZIKV resulted in 37 and 42% mortality, respectively, over a course of 30 days of infection. ZIKV replication was detected in the brain, testes, spinal cords, kidney and spleen, although no histopathological lesions were seen. Infection of pregnant STAT2 knockout (KO) hamsters, but not immunocompetent hamsters, led to ZIKV infection in placental and foetal brains, but no adverse phenotype in pups. Taken together, these studies indicate that wild-type hamsters are not susceptible to infection, but STAT2 KO animals are valuable for some studies.

Swine
Swine are used as a model for biomedical research because they share similarities in several aspects of human anatomy, physiology, genetics and immune response. Regarding their immunity, pigs closely resemble humans in more than 80% of immunological parameters, as opposed to less than 10% in mice [123]. In addition, pigs are susceptible to several flaviviruses, including dengue virus (DENV), WNV and JEV [124]. Based on this, Darbellay et al. experimentally infected neonatal piglets with a 2015 Puerto Rican strain of ZIKV using three different inoculation routes: intracerebral, intradermal and intraperitoneal. They found that 2 out of 11 piglets inoculated intracerebrally, a non-natural route of infection, exhibited leg weakness, ataxia and tremor. ZIKV infection of piglets also resulted in seroconversion and low-titre viraemia, viruria and virus replication in internal organs, demonstrating that newborn pigs can be used as models to study some aspects of ZIKV biology (Table 4). An independent study using 3-month-old pigs did not detect viraemia or overt clinical signs, albeit the animals did seroconvert [121]. From these initial studies, it seems that pigs can serve as reservoirs for ZIKV, but not as amplifying hosts, given the low viraemia following the infection. However, additional studies using a larger number of animals are needed to clarify the role of pigs in the epidemiology of ZIKV.

Experimental in utero infection of conventional swine with ZIKV at 50 gestation days (i.e. mid-gestation) resulted in persistent infection in porcine conceptuses and impaired health in porcine offspring characterized by weak piglets, delayed feeding, lower neonatal body length and weight than controls, seizure-like activities, splayed back leg, reduced growth rate and aggressive behaviour [125]. A recent study inoculated pregnant sows with ZIKV in utero at 50 gestation days and euthanized them at 4 weeks after inoculation, which is comparable to the end of the second trimester of human pregnancy, and examined the foetuses [126]. The sows remained healthy during the experiment, but at necropsy 2 out of 15 inoculated foetal piglets were found to have microencephaly and all inoculated foetuses presented mild to severe neuropathology, characterized by neuronal depletion in the cerebral cortices of various lobes (Table 5). Although useful, their large size and high cost, and the lack of disease signs exhibited by the majority of infected animals, limit the utility of swine for widespread use in ZIKV research.

Non-human primates
Nonhuman primates (NHPs) are the most closely related animals to humans and are therefore the preferred human surrogates in ZIKV studies. NHPs are natural hosts for ZIKV and recapitulate several aspects of the disease in humans. In fact, the first described ZIKV-susceptible animal model was the rhesus monkey, which had historical importance for the discovery of ZIKV in 1947. Mice intracerebrally inoculated with viraemic rhesus monkey sera developed clinical disease, but mice and monkeys inoculated with the same virus intraperitoneally had no overt disease [127]. Since the emergence of ZIKV in the Americas, several groups have carried out experiments in NHPs to understand pathogenesis and transmissibility and develop countermeasures [128–143]. NHP models are essential for advancing ZIKV vaccine and drug candidates into clinics. Table 6 summarizes the studies that have used NHPs for ZIKV. Although NHP models are the most similar to human beings, the need for special animal facilities and their high cost and limited availability, together with the ethical issues associated with their use, limit the applications of this model for ZIKV studies.
Rhesus macaque (Macaca mulatta)

Rhesus macaques have been the most widely used NHP model for ZIKV. Most studies have used needle infection by the subcutaneous route to mimic mosquito bites. In these experiments, ZIKV infection in non-pregnant animals resulted in either subclinical infection or mild clinical signs of disease, such as rash, fever and conjunctivitis. Viraemia and virus excretion in several body fluids, such as urine, saliva, tears, semen, vaginal secretions and cerebrospinal fluid, have been reported. ZIKV has also been detected in several organs, where it resulted in gross and histopathological changes, with a marked tropism to the CNS [128–135]. ZIKV can persist in the CNS, lymph nodes and other immune privileged sites of rhesus monkeys for weeks after virus has been cleared from peripheral blood, urine and mucosal secretions [144]. ZIKV infection resulted in robust innate and humoral and cellular adaptive responses in these animals, which were protected from subsequent challenge with homologous or heterologous strains [131–133, 136, 137]. This monkey species has been useful in characterizing the innate and adaptive immune responses after ZIKV infection. In one study, Hirsch and coworkers infected pregnant rhesus macaques and found that ZIKV causes placental dysfunction and immunopathology. Within 5 days p.i., all infected dams showed innate immune cell activation, as demonstrated by the presence of CD169+ staining within monocytes/macrophages, myeloid DCs and NK subsets. Infected dams displayed marked activation of DCs and NK cells which peaked at 70 and 85 days p.i. Proliferation of CD8 T-cell and B-cell was also detected, with maximum responses seen in the first two weeks post infection. ZIKV-specific maternal antibodies were detected as early as 6 days p.i. and the antibody titres increased through 28 days p.i. Serum collected from dams and foetuses at 85 days p.i. showed neutralizing activity [136]. The evaluation of three vaccine platforms (inactivated vaccine, plasmid DNA vaccine or a rhesus adenovirus serotype 52 vector-based vaccine) led to the development of neutralizing antibodies and full protection of the rhesus macaques against challenge [129]. The infection of pregnant animals resulted in long-lasting viraemia, vertical transmission and microcephaly in foetuses [137]. The viraemia in pregnant animals was longer than that for non-pregnant animals [128, 143]. A recent study demonstrated that the infection of pregnant rhesus monkeys early in pregnancy recapitulates many lesions that are characteristic of congenital Zika syndrome (CZS), including foetal loss, smaller brain size, and microscopic brain pathology characterized by microcalcifications, necrosis, vasculitis, haemorrhage, glossis and apoptosis of neuroprogenitor cells. The viraemia in animals infected early in pregnancy was longer (28–70 days) than that in animals infected later in pregnancy (14–42 days) [143].

A single study that inoculated ZIKV intravenously reported no clinical disease, but the animals developed short-lived viraemia, viruria and virus excretion in saliva. ZIKV was present in the lymph nodes and spleen and in cardiopulmonary, gastrointestinal, integument and genitourinary tissues, in the absence of major histopathological changes [135]. Direct inoculation of high-dose ZIKV directly to the tonsils resulted in viraemia, but saliva from infected monkeys inoculated in the palatine tonsils or conjunctiva or nasally did result in infection, suggesting that the transmission risk from saliva is low [139]. Animals inoculated through either the vaginal or the rectal route had overt clinical disease, but viraemia was detected in 50 and 100% of the macaques inoculated by these routes, respectively [138].

Cynomolgus macaques (Macaca fascicularis)

The ban on exporting rhesus monkeys from India has reduced the availability of these animals and stimulated research using other NHP species. Cynomolgus macaques (Macaca fascicularis) are closely related to rhesus monkeys, but they are smaller and therefore easier to handle and maintain in animal facilities.

Similar to rhesus monkeys, subcutaneous inoculation of these animals with the PRVABC59 strain resulted in viraemia and viral detection in the lymph nodes, CNS and male (seminal vesicles, testes, prostate) and female genital tracts (uterus, ovaries) [132]. Intravaginal and intrarectal inoculation resulted in viraemia in 50 and 100% of inoculated animals, respectively, and no clinical disease [138]. Infections carried with the Asian and African ZIKV strains suggested that Cynomolgus monkeys appear to be susceptible only to infection with ZIKV isolates of the Asian lineage [134], although these results have not been reproduced using different African isolates of ZIKV.

Pig-tailed macaque (Macaca nemestrina)

The pigtail macaque (Macaca nemestrina) has been known to be susceptible to several viruses from the family Flaviviridae, including DENV, JEV and HCV. ZIKV that was inoculated subcutaneously in pregnant pigtail monkeys did not cause overt clinical disease, but the foetuses developed brain lesions characterized by cerebral white matter hypoplasia, periventricular white matter gliosis, and axonal and ependymal injury, all of which are associated with the presence of ZIKV RNA [130].

Marmoset (Callithrix jacchus)

The common marmoset (Callithrix jacchus) is a small-sized primate from the Americas that has been regarded as a relevant and convenient experimental model for investigating ZIKV pathogenesis. Intramuscular infection of these animals with the MR766 strain (Uganda/1947) resulted in no overt clinical disease, but ZIKV persisted in body fluids such as semen and saliva for longer periods of time than in serum. Infection with this non-contemporary African strain elicited strong neutralizing antibodies and antiviral responses, and complete protection against a heterologous challenge with a recent Asian lineage strain isolated in Brazil [140].

A second study by the same research group in pregnant marmosets reported no clinical disease in these animals upon infection with the SPH2015 strain (Brazil/2015). However, the animals developed viraemia, viruria and had...
Table 6. Non-human primate models for ZIKV

<table>
<thead>
<tr>
<th>Animal species</th>
<th>ZIKV strain (country/year)</th>
<th>Route of infection/dose</th>
<th>Major findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus macaque <em>(Macaca mulatta)</em></td>
<td>H/PF/2013 (French Polynesia/2013)</td>
<td>Subcutaneous /10^4/10^5 p.f.u.</td>
<td>Viraemia in non-pregnant and pregnant animals for 21 days and for up to at least 57 days, respectively. Virus was present in saliva, urine and cerebrospinal fluid. Homologous challenge at 10 weeks after the initial infection resulted in protection from disease and virus replication</td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>Brazil/ZKV/2015 (Brazil/2015)</td>
<td>Subcutaneous/10^4 p.f.u.</td>
<td>Animals were immunized with either an inactivated vaccine, a plasmid DNA vaccine or a rhesus adenovirus serotype 52 vector-based vaccine. Vaccinated animals developed neutralizing antibodies and were fully protected from challenge</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>GZ01/2016 (China/2016)</td>
<td>Subcutaneous/10^4 p.f.u.</td>
<td>Fever, viraemia and ZIKV RNA excretion in urine, saliva and lacrimal fluid. Necropsy of two infected animals revealed systemic infections involving the CNS and viseral organs. Robust humoral and cellular response was detected in infected animals</td>
<td>[131]</td>
</tr>
<tr>
<td></td>
<td>PLCal_ZV (Canada/2014) and PRVABC59 (Puerto Rico/2016)</td>
<td>Subcutaneous/10^4 p.f.u.</td>
<td>High-level viraemia and viral detection in saliva, urine, cerebrospinal fluid (CSF) and semen (but only transiently in vaginal secretions); increase in the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and creatinine phosphatase. Animals were protected from heterologous ZIKV infection</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>MR766 (Uganda/1947) and H/PF/2013 (French Polynesia/2013)</td>
<td>Subcutaneous/10^4 to 10^6 p.f.u.</td>
<td>Immunity elicited by African lineage ZIKV protects against subsequent infection with Asian lineage ZIKV; robust cellular and humoral immunity to ZIKV</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>PRVABC59 (Puerto Rico/2016)</td>
<td>Subcutaneous/10^4 to 10^6 p.f.u.</td>
<td>Transient plasma viraemia and viruria from 1 to 7 days p.i.; rash, fever and conjunctivitis; strong adaptive immune response to ZIKV, although systemic cytokine response was minimal; virus detection in peripheral nervous tissue, multiple lymphoid tissues, joints, and the uterus of the necropsied animals at 7 days p.i.</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td>H/PF/2013 (French Polynesia/2013)</td>
<td>Subcutaneous or oral/10^4 p.f.u.</td>
<td>Application of high-dose ZIKV directly to the tonsils resulted in viraemia. Animals infected either to the palatine tonsils, conjunctive or nasal passages with saliva from monkeys infected subcutaneously did not become infected</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td>Brazil/ZKV/2015 (Brazil/2015)</td>
<td>Intravenous/10^4 p.f.u.</td>
<td>Animals had no clinical disease; short-lived viraemia that cleared as neutralizing antibody developed; ZIKV RNA detected in urine and saliva; virus present in lymph nodes, spleen, cardiopulmonary, gastrointestinal, integument and genitourinary tissues</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>H/PF/2013 (French Polynesia/2013)</td>
<td>Subcutaneous or oral/10^4 p.f.u.</td>
<td>Pregnant macaques had viraemia from 11 to 70 days p.i.; head circumference (HC) in all foetuses was between one and three SD below the mean and ZIKV RNA was detected in tissues from all foetuses, suggesting consistent vertical transmission</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td>PRVABC59(Puerto Rico/2016) or Brazil/ZKV/2015 (Brazil/2015)</td>
<td>Subcutaneous/10^4 to 10^7.5 f.u.</td>
<td>Persistence in central nervous system, lymph nodes and coloerctal tissues for weeks after virus has been cleared from peripheral blood, urine and mucosal secretions</td>
<td>[144]</td>
</tr>
<tr>
<td></td>
<td>ArD 41525 (Senegal/1984)</td>
<td>Intravaginal or intrarectal/10^4 p.f.u.</td>
<td>No overt clinical signs; viraemia was detected in 50 and 100 % of macaques inoculated intravaginally or intrarectally, respectively</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td>Brazil/ZKV/2015 (Brazil/2015)</td>
<td>Subcutaneous/10^4 p.f.u.</td>
<td>Infection of pregnant monkeys recapitulates many lesions that are characteristic of CZS, including consistent neuropathology in the foetal brain and spinal cord. Pregnant animals infected early in pregnancy exhibited prolonged viraemia (28–70 days) compared with dams infected later in pregnancy (14–42 days)</td>
<td>[143]</td>
</tr>
<tr>
<td>Cynomolgus macaques <em>(Macaca fascicularis)</em></td>
<td>PRVABC59 (Puerto Rico/2016)</td>
<td>Subcutaneous/10^5 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Viraemia, viral detection in lymph nodes, CNS and male (seminal vesicles, testes, prostate) and female genital tracts (uterus, ovaries)</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>ArD 41525 (Senegal/1984)</td>
<td>Intravaginal or intrarectal/10^4 p.f.u.</td>
<td>No overt clinical signs; viraemia was detected in 50 and 100 % of macaques inoculated intravaginally or intrarectally, respectively</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td>PRVABC59(Puerto Rico/2016) or FSS13025 (Cambodia/2010) or IBH30656 (Nigeria/1968)</td>
<td>Subcutaneous/10^4 to 10^7 p.f.u.</td>
<td>RVABC59 strain induced viraemia detectable up to day 10 and moderate viral in testes, urine and saliva. FSS13025 strain induced shorter and lower viraemia compared to PRVABC59 and had detectable viral loads in testes but not in urine and saliva. IBH30656 failed to establish infection</td>
<td>[134]</td>
</tr>
</tbody>
</table>
spontaneous pregnancy loss at 16–18 days p.i., with extensive active ZIKV replication in the placenta and foetal neurocellular disorganization indicating a disruption in the development and neuronal migration patterns of the cerebral cortex, as seen in humans [141].

Other vertebrate animal models

Early studies with the prototype MR766 strain in rabbits and cotton rats has shown that these animals do not develop clinical signs of infection after intracerebral inoculation of late-passage mouse brain virus, but do seroconvert [116]. In addition to serological evidence for natural infection, some African bat species supported replication and developed clinical signs of disease upon experimental infection with ZIKV. However, studies with contemporary ZIKV strains in bat species from the New World are needed to better elucidate the role of bats in ZIKV ecology [145–147].

Recently, Ragan et al. infected several animal species from North America with ages ranging from neonates to adults. The species investigated included armadillos, cottontail rabbits, goats, mink, chickens, pigeons, ground hogs, deer mice, cattle, raccoons, ducks, Syrian golden hamsters, garter snakes, leopard frogs, house sparrows and pigs. Animals were infected with ZIKV by the subcutaneous and intradermal routes with a high virus dose (10^5 p.f.u.). Low-level viremia was found only in frogs and armadillos. Neutralizing antibodies were detected in goats, rabbits, ducks, frogs and pigs, suggesting these animals are unlikely to act as animal reservoirs or good models to reproduce ZIKV disease [121].

Before the ZIKV epidemics reported in Yap Island (2007) and in French Polynesia (2013), very few studies registered experimental infections in mosquitoes. These studies were mainly performed to demonstrate the vectorial competence of mosquito species in order to identify or incriminate a mosquito species as potential vectors. *Ae. aegypti* has been used as the major experimental model, as it is a mosquito species that is easy to colonize and maintain in insectaries. It was the first mosquito species to be used in an artificial feeding system for studying ZIKV infections (148Boorman and Porterfield, 1956). *Ae. aegypti* was employed to feed directly on a Zika patient through the exposure of the left forearm to a batch of mosquito females in order to demonstrate ZIKV transmission by mosquitoes [149]. Most of the studies use an artificial membrane feeder to infect mosquitoes and study the spread of ZIKV in the mosquito organs. Usually, the midgut and salivary glands or mosquito saliva are collected to detect and quantify the presence of ZIKV particles. In addition, ZIKV can be inoculated via intrathoracic injection [150]. *Ae. aegypti* artificially infected with ZIKV through blood meal successfully transmitted ZIKV to BALB/c mouse upon feeding on the ears of these animals, an approach that allows evaluation of the development of infection in and transmission from mosquitoes using a transmission process that resembles the natural process [151]. *Ae. aegypti* that were allowed to feed on infected AG129 mice acquired ZIKV in their midguts, but transmission from these mosquitoes depended largely on the colony of *Ae. aegypti* used, suggesting that genetic differences play an important role in vector competence for ZIKV [150].

A recent debate about the possible role that another mosquito species, *Culex quinquefasciatus*, could play in ZIKV transmission [152] inspired several groups to investigate the vector competence of mosquito species from the genera *Aedes* and *Culex*. Several studies using different combinations of ZIKV strains and mosquito colonies or populations failed to demonstrate ZIKV transmission by *Culex* species [153–155]. However, Guo et al. successfully demonstrated
the transmission of ZIKV by Cx. quinquefasciatus to mice [156]. In addition, Guedes et al. photographed ZIKV mature particles in the salivary glands of this mosquito species after 7 days p.i. [157]. Further field studies reported Cx. quinquefasciatus naturally infected with ZIKV [158], confirming that it is possible that other species besides Ae. aegypti are transmitting ZIKV in urban environments. Further investigations are required to determine the primary, secondary or occasional vectors of ZIKV during outbreaks. Care should be taken in interpreting transmission studies using laboratory-reared mosquitoes. Furthermore, although the virus maybe found in certain mosquito species, this does not mean the the virus will be transmitted by that species under natural conditions.

Thus, mosquito models fed using the available systems of artificial infection could be better exploited, for instance in studies to understand the pathogen-vector-host interactions, in order to identify the immune response triggered by distinct mosquito species against the viral strains. This response, together with receptor molecules, is the basis for a mosquito that is refractory to viral infection and could be used in future studies to develop new strategies for ZIKV control.

MODELS TO STUDY ANTIBODY-DEPENDENT ENHANCEMENT

Antibody-dependent enhancement (ADE) is a phenomenon by which non-neutralizing antibodies enhance viral entry and replication in host cells, resulting in increased viral load and exacerbation of clinical disease. ADE is a well-studied mechanism in DENV pathogenesis and is thought to occur through the formation of infectious virus–antibody immune complexes (ICs) that bind to Fc gamma receptors (FcγR) expressed on permissive cells such as DCs and monocytes/macrophages [159]. The enhancement of ZIKV infection by DENV antiserum was first described in 1987 [160], and has been evaluated by many groups recently, given the possible impact of ADE on ZIKV pathogenesis, vaccine development and immunotherapy [17, 161–165].

Typical in vitro assays to study ADE consist of incubating ZIKV with serially diluted test serum (or antibodies) and then infecting a cell line that expresses the FcγR. ADE is then defined as a significant increase in virus titres obtained after incubation with test serum relative to controls. Several cell lines have been used to study ZIKV ADE activity, including the murine macrophage-like P388D1 cell line [160], FcγR-expressing BHK cell lines [166], the human erythroleukemic K-562 cell line [162, 165, 167], the human macrophage cell line U937 [17], the human monocytic cell line THP-1 [161] and primary human macrophages [168].

ADE of ZIKV infection has been evaluated in vivo using immunodeficient mice and monkeys. Bardina et al. were able to reproduce ADE in vivo by using Stat2−/− knockout C57BL/6 mice and convalescent plasma from DENV- and WNV-infected individuals and then infecting these animals 2 hours later with the ZIKV strain PRVABC59. They found that pretreatment with anti-DENV or anti-WNV plasma resulted in more elevated body temperature, higher viraemia and viral burden in the spinal cord and testes (but not in the brains, ovaries and eyes), increased weight loss, higher mortality and enhanced clinical symptom scores relative to controls. Their study also showed that in vivo ADE occurs optimally at low concentrations of ZIKV-reactive IgG, while high levels may be protective [167]. In fact, treatment of type I interferon receptor-deficient (IFNAR−/−) mice with a cross-reactive dengue human monoclonal antibody (mAb) protected adult non-pregnant mice from ZIKV-induced weight loss and mortality. Treatment of pregnant mice infected with ZIKV with mAb also significantly reduced the viral titres in the placenta and foetal organs and abolished virus-induced foetal growth retardation [165].

Recently, a study using two groups of rhesus monkeys (one naive to flavivirus infection and the other previously exposed to DENV almost 3 years earlier) demonstrated that while ADE could be confirmed in vitro, preexisting DENV immunity did not result in exacerbated ZIKV disease [163]. These findings were supported by a human cohort study of ZIKV-infected patients previously exposed to dengue [164]. However, more studies using a larger number of animals and including patients from different epidemiological settings are needed to better establish the role of ADE on ZIKV-induced disease.

CONCLUSION AND PERSPECTIVES

Tremendous advances have been made in the development of in vitro and in vivo models that recapitulate many aspects of ZIKV biology and disease in humans. Infection by contemporary ZIKV strains has been characterized in a variety of cell culture systems and animal models, and this has already contributed to advancements in the fields of viral pathogenesis, epidemiology, vaccinology and antiviral discovery. However, each of these models has limitations that must be considered in the design and interpretation of experiments, and in the extrapolation of experimental results to humans. Sharing of these research models among different laboratories will ensure data reproducibility and accelerate the discovery and development of new products and processes. The continuous development and characterization of research models is essential for a better understanding of ZIKV biology and the translation of research findings from the bench to the clinic.

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Conflicts of interest
The authors declare that there are no conflicts of interest.
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